REMARKS

Claims 1-9, 11, 13-15 and 20 are pending in the application. Claims 1-9, 11, and 20 have been cancelled. Claims 13-15 have been amended and new claims 21-60 have been added. Support for the claim amendments and new claims may be found throughout the specification, including the claims as originally filed. No new matter has been added.

In particular, amendments to claims 13, 14 and 15 may be found on pages 15, 55, and 74-77. Support for the phrase "or the complement thereof" in claim 13 and the phrase "SEQ ID No: 15" may be found on page 17, and Figure 4 of the specification respectively. Support for the claim amendment "including a wash step in 0.2x SSC at 65°C" in claim 14 may be found on page 55.

Support for new claims 21-28 may be found in pages 4, 14, 18, 52-55, 74-77 and Figure 4.

Support for new claims 29-36 may be found on pages 25, 46-50, 58-60, 77.

Support for new claims 37-48 may be found on pages 21, 62, 77

Support for new claims 49-60 may be found in pages 13-14, 19, 23-25, 46-50, 69-74.

Amendment of claims should in no way be construed as an acquiescence to any of the Examiner's rejections. The amendments to the claims are being made solely to expedite prosecution of the present application. Applicants reserve the right to further prosecute claims drawn to all subject matter disclosed in the instant patent application or in a continuation hereof.

Amendments to the abstract of the disclosure have been made to address the Examiner's objection to it. Amendments to the specification have also been made to incorporate sequence identification numbers in accordance with the sequence listing submitted September 7, 2001. No new matter has been added by these amendments.

The Examiner's remarks in the last Office Action are addressed below. It is believed that the amended claims and all dependent claims, taken in light of the remarks made herein, meet all criteria for patentability.

CLAIM OBJECTIONS

The Examiner has objected to claim 7 because it recites the enzymatic activities of nonelected inventions. The Examiner has also objected to claim 20 because "it depends from a claim directed to a nonelected invention". Finally, the Examiner has objected to claim 8 because "the sequence is not referred to by use of the sequence identifier". Claims 7, 8 and 20 are herein cancelled without prejudice, rendering the instant objections moot. Applicants reserve the right to further prosecute the subject matter of these claims in this or a subsequent related patent application.

CLAIM REJECTIONS

Rejection of claims under 35 U.S.C. §112, first paragraph

Written Description

The Examiner has rejected claims 1-7, 9, 11, 14-15 and 20 as containing "subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention." Specifically, the Examiner has pointed out that,

> "[t]he claims are drawn to a transgenic plant comprising any heterologous gene of unknown function from any unspecified marine vascular plant, including transgenic plants comprising any heterologous gene of unknown function from *Zostera marina*, any heterologous zosteric acid biosynthesis gene from any unspecified marine vascular plant, any heterologous saline-resistance gene from any unspecified marine vascular plant, any heterologous hypoxia-resistance gene from any unspecified marine vascular plant, and any nucleic acid from any source and of any sequence which hybridizes to SEQ ID NO. 1 under conditions of unspecified stringency."

The Examiner has further objected to the claims as they are

> "drawn to a transgenic plant comprising any heterologous nucleotide sequence encoding a zosteric acid biosynthetic function from any organism, including any heterologous nucleotide sequence encoding a sulfotransferase activity, a transgenic comprising any heterologous nucleotide sequence encoding a saline-resistance function from any organism, and a transgenic plant comprising any heterologous nucleotide sequence encoding a saline-resistance function from any organism, and a transgenic plant possessing an antifouling genetic trait and transformed with a cDNA sequence obtained from a marine vascular plant that hybridizes to a nucleic acid encoding a sulfotransferase".

Applicants respectfully traverse this rejection.

The Examiner has acknowledged that the specification "discloses the cloning from *Zostera marina* of a nucleotide sequence encoding a polypeptide having a moderate level of homology to known flavonol sulfotransferases" as set forth in Figure 4 (SEQ ID NO: 15). The Examiner further acknowledges that the specification also discloses that the polypeptide encoded by the cloned nucleotide sequence has sulfotransferase activity.

Claims 1-7, 9, 11 and 20 have been cancelled without prejudice, rendering this rejection moot. Claim 14 as amended, is drawn to an isolated nucleic acid which hybridizes under stringent conditions including a wash step in 0.2x SSC at 65°C to the nucleic acid consisting of the nucleotide sequence set forth in SEQ ID NO: 15. Claim 15 as amended, is drawn to an isolated nucleic acid of claim 14 which encodes a sulfotransferase.

Applicants respectfully submit that the specification provides adequate written description for these claims as well as the new claims. As acknowledged by the Examiner, the specification teaches the nucleotide and amino acids sequences of a *Zostera marina* sulfotransferase, i.e. SEQ ID NO: 15 and SEQ ID NO: 16 respectively. Thus a person of skill in the art would have recognized that Applicants were in possession of the claimed invention at the time of filing.

Enablement

The Examiner has rejected claims 1-9, 11, 13-15 and 20 because "[t]he claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention". Specifically, the Examiner acknowledges that the specification discloses "the cloning from *Zostera marina* of a nucleotide sequence encoding a polypeptide having a moderate level of homology to known flavonol sulfotranferases" and that "the polypeptide encoded by the cloned nucleotide sequence has sulfotransferase activity." However, the Examiner states that

> the specification does not teach how to make or use any other heterologous genes associated with genes associated with any other function from any other marine vascular

> plant or from any other source. Furthermore, the specification does not disclose the use of any of the *Zostera marina* nucleotide sequence in a transgenic plant, or the effect of expressing any nucleotide sequence in a transgenic plant on traits such as saline-resistance or hypoxia-resistance or antifouling.

Applicants respectfully traverse this rejection.

Claims 1-9, 11 and 20 have been canceled without prejudice, rendering their instant rejection moot.

Claim 13, as amended, is drawn to an isolated nucleic acid comprising the sequence of SEQ ID NO: 15 or a subsequence of at least 50 nucleotides of SEQ ID NO: 15 or the complement thereof. Claim 14, as amended, is drawn to an isolated nucleic acid which hybridizes under stringent conditions including a wash step in 0.2x SSC at 65°C to the nucleic acid consisting of the nucleotide sequence set forth in SEQ ID NO: 15. Claim 15, as amended, is drawn to an isolated nucleic acid of claim 14 which encodes a sulfotransferase.

It is the Examiner's position that "[g]uidance for making and using the claimed invention is necessary because it is unpredictable whether expressing a heterologous nucleotide sequence, such as a heterologous nucleotide sequence encoding a sulfotransferase activity, in a transgenic plant would confer a useful trait, such as saline-resistance or hypoxia-resistance or antifouling," because "[t]he ability of a heterologous nucleotide sequence to confer a useful trait on a transgenic plant is unpredictable because traits such as saline-resistance or hypoxia-resistance or antifouling are multigenic traits that require the coordinated activity of multiple proteins," and that "[t]he expression of a single polypeptide encoded by a single heterologous nucleotide sequence may not affect a multigenic trait in a transgenic plant unless that polypeptide participates in a rate limiting step of the process that confers the trait." Applicants respectfully submit that the claimed sulfotransferase converts p-hydroxycoumaric acid into zosteric acid, which provides antifouling characteristics (see Figure 1). For example, Applicants have shown that the sulfotransferase from *Zostera marina* catalyzes the transfer of sulfate to the standard phenol substrate quercetin (see page 77, second and third paragraphs). This substrate is an accepted substitute for coumaric acid (See Exhibit A (Varin et al. (1992) *Proc. Natl. Acad. Sci.*, 89:1286)). In addition, it is well known that zosteric acid (also known as p-(sulphooxy) cinnamic acid or p-coumaric acid sulphate) provides antifouling characteristics. See Exhibit B

(Todd et al. (1993) *Phytochemistry* 34:401), Exhibit C (U.S. Patent No. 5,607,741), Exhibit D (U.S. Patent No. 5,384,176 and Example 5.3, starting at page 79)).

Applicants further submit that no other proteins are necessary for providing the antifouling trait, provided that p-hydroxycoumaric acid is present in the plant in which the sulfotransferase gene is inserted, i.e., the sulfotransferase is the only protein necessary for converting coumaric acid into zosteric acid. Furthermore, it is well known that essentially all plants express sufficient levels of p-hydroxycoumaric acid that expression of a sulfotransferase gene will convert into sufficient quantities of zosteric acid to provide antifouling characteristics to the transgenic plant. Indeed, coumaric acid is involved in pathways producing amino acids and lignins and therefore most plants have a sufficient flux of coumaric acid. For example, Table 4-3 in Exhibit E (Robinson, T. (1980) *The Organic Constituents of Higher Plants (Their Chemistry and Interrelationships)* 5th ed., Cordus Press) and Exhibit F (Zapata et al. (1979) *Aquatic Botany* 7:307-317) describe that presence of coumaric acid is widespread in plants.

The Examiner further states that "the level of activity of any enzyme is dependent on the availability of specific molecules that positively and negatively regulate that enzyme's activity, which could vary within a species between cell types and between species." Applicants respectfully submit that the claimed sulfotransferase is part of a class of sulfotransferases that are not subject to positive or negative regulation, and that its expression in a plant is sufficient to convert p-hydroxycoumaric acid into zosteric acid.

The Examiner also states that "[b]ecause different sulfotransferase enzymes require different substrates and reaction conditions in order to be biologically active, and because it is unknown which plant or plant cell types would provide the appropriate substrates and reaction conditions for a sulfotransferase enzyme to function in such a way as to cause saline-resistance or antifouling, the claimed invention is not enabled by the specification in the absence of further guidance or example." Applicants respectfully submit that, as set forth above, the claimed sulfotransferase converts p-hydroxycoumaric acid into zosteric acid, and that most plants contain p-hydroxycoumaric acid, so that expression of the claimed sulfotransferase in plants would result in sufficient zosteric acid produced to provide the plant with antifouling characteristics.

Thus, Applicants respectfully request reconsideration and withdrawal of this rejection.

Rejection of claims under 35 U.S.C. §112, second paragraph

The Examiner has rejected claims 1, 3, 4, 5, and 13-15 and claims dependent thereon, "as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention." Applicants respectfully traverse this rejection.

Claims 1, 3, 4, and 5 are herein cancelled without prejudice, rendering the instant rejection moot with respect to these claims.

Claims 13-15 have been rejected as being "indefinite in referring to SEQ ID NO: 1 as a nucleic acid, as SEQ ID NO: 1 in the sequence listing is an amino acid sequence". Applicants have amended claims 13-14 to refer correctly to SEQ ID NO: 15 which is a nucleotide sequence. Thus reconsideration and withdrawal of these rejections is respectfully requested.

Rejection of claims under 35 U.S.C. §101

The Examiner has rejected claims 13-15 because "the claimed invention is directed to non-statutory subject matter" and "as written, do not sufficiently distinguish over nucleic acids as they exist naturally because the claims do not point out any non-naturally occurring products". The Examiner points out that "[t]he claims should be amended to indicate the hand of the inventor, e.g., by insertion of "Isolated" or "Purified"." Applicants have amended the claims to recite the term "isolated" in accordance with the term used by the Examiner. It is believed that the present rejection has been obviated and that the amended claims comply fully with 35 U.S.C. §101.

Rejection of claims under 35 U.S.C. §102

Claims 1, 2, 3, 6, 7, 14, 15, and 20 are rejected under 35 U.S.C. §102(b) as being anticipated by Varin et al. Applicants respectfully traverse this rejection.

Claims 1, 2, 3, 6, 7, and 20 have been cancelled rendering the instant rejection moot with respect to these claims.

Claim 14 as amended, is drawn to an isolated nucleic acid which hybridizes under stringent conditions including a wash step in 0.2x SSC at 65°C to the nucleic acid consisting of the nucleotide sequence set forth in SEQ ID NO: 15. Claim 15 as amended, is drawn to the isolated nucleic acid of claim 14, which encodes a sulfotransferase.

The Examiner relies on Varin et al. as teaching "transgenic canola, potato and tobacco plants comprising a heterologous nucleotide sequence encoding a flavonol sulfotransferase from *Flaveria chloraefolia*".

To anticipate a claim, a reference must teach each and every element of the claim. Varin et al. does not teach an isolated nucleic acid which hybridizes under stringent conditions including a wash step in 0.2x SSC at 65°C to the nucleic acid consisting of the nucleotide sequence set forth in SEQ ID NO: 15 or an isolated nucleic acid which encodes a sulfotransferase (see e.g. page 76 and Figure 5 of the specification). Accordingly, because Varin et al. does not teach each and every element of claims 14 and 15, Applicants respectfully request reconsideration and withdrawal of these rejections under 35 U.S.C. §102(b).

Claims 1, 2, 4, and 9 are further rejected under 35 U.S.C. §102(b) as being anticipated by Xu et al. Applicants respectfully traverse this rejection. Claims 1, 2, 4 and 9 are herein withdrawn without prejudice, rendering the instant rejection moot with respect to these claims. It is believed that the new claims and amended claims are not anticipated by Xu et al. as none of the claims teach a "transgenic rice plant comprising a heterologous nucleotide sequence encoding a HVA1 saline resistance function from barley."

Claims 1, 2, 5, and 11 are further rejected under 35 U.S.C. §102(b) as being anticipated by Hoeren et al. Applicants respectfully traverse this rejection. Claims 1, 2, 5 and 11 are herein withdrawn without prejudice, rendering the instant rejection moot with respect to these claims. It is believed that the new claims and amended claims are not anticipated by Hoeren et al. as none of the claims teach "transgenic pea plants comprising a heterologous nucleotide sequence encoding an AtMYB2 hypoxia-resistance function from *Arabidopsis*.

Thus, Applicants respectfully request reconsideration and withdrawal of these rejections under 35 U.S.C. §102(b).

CONCLUSION

For the foregoing reasons, Applicants respectfully request reconsideration and withdrawal of the pending rejections. Applicants believe that the claims now pending are in condition for allowance, and notification of such is respectfully requested.

The Commissioner is hereby authorized to credit any overpayment or charge any deficiencies to Deposit Account Number **06-1448, Reference CEA-007.01**

If, for any reason, a telephonic conference with the Applicant would be helpful in expediting prosecution of the instant application, the Examiner is invited to call Applicants' Agent at the telephone number provided below.

Respectfully submitted,
FOLEY HOAG LLP

December 17, 2003

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Exhibit A

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Biochemistry

Molecular characterization of two plant flavonol sulfotransferases

(Flaveria chloraefolia)

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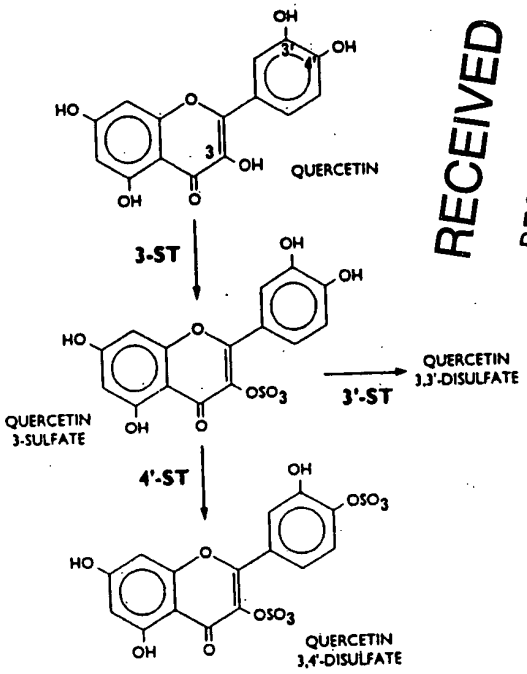
Communicated by Eric E. Conn, November 7, 1991

ABSTRACT cDNA clones coding for flavonol 3- and 4'-sulfotransferases (STs) were isolated by antibody screening of a cDNA expression library produced from poly(A)$^+$ RNA extracted from terminal buds of *Flaveria chloraefolia*. Sequence analysis revealed full-length cDNA clones with open reading frames of 933 and 960 base pairs, which encode polypeptides containing 311 and 320 amino acids, respectively. This corresponds to a molecular mass of 36,442 Da for the 3-ST and 37,212 Da for the 4'-ST. Expression of these clones in *Escherichia coli* led to the synthesis of β-galactosidase–ST fusion proteins having the same substrate and position specificities as those for the 3- and 4'-flavonol ST enzymes isolated from the plant. Comparison of the deduced amino acid sequence of the two clones revealed an overall identity of 69% in 311 amino acid residues. The two flavonol STs of *F. chloraefolia* also shared significant sequence similarities with steroid and aryl STs found in animal tissues and with the senescence marker protein 2 isolated from rat liver, suggesting an evolutionary link between plant and animal STs.

Plants accumulate a variety of natural products that are synthesized in response to environmental stimuli and genetically programmed developmental signals. Of these metabolites, flavonoid compounds are probably the most ubiquitous. Various flavonoids have been recognized as important stress metabolites that are synthesized in response to UV irradiation (for review, see ref. 1) and microbial attack (2–5). More recently, a number of flavonoids have been reported to act as signaling molecules that are involved in the induction/inhibition of early nodulation genes in *Rhizobium* (6) and as regulators of polar auxin transport (7). A class of flavonoids esterified with sulfate groups have recently been reported to be of common occurrence in a number of plant families (8, 9). Most of these compounds are mono- to tetrasulfate esters of common flavones and flavonols or their methyl ethers and, less commonly, of their glycosylated derivatives.

The functional significance of flavonoid sulfates in plant tissue is not clear. Apart from their possible involvement in detoxification of reactive hydroxyl groups, their accumulation in plants growing in saline or marshy habitats suggests a role in the sequestering of sulfate ions (8). Recently, sulfated phenol glucosides called turgorins were shown to be responsible for the seismonastic and gravitropic movements of plants (10), whereas sulfated lipooligosaccharides induced root nodule organogenesis in the interaction between *Rhizobium meliloti* and alfalfa (11). In both cases the presence of the sulfate group was required for biological activity, suggesting its requirement in recognition and signaling processes.

In animal tissues, enzymatic sulfation is considered an important reaction in the detoxification of endogenous metabolites and xenobiotics. Investigation of sulfate metabolism in these tissues led to the recognition of a number of sulfotransferases (STs) with specificity toward a variety of metabolites including arylamines, phenols, steroids, and bile acids (12–14). cDNA clones coding for four of these enzymes have been isolated and characterized (15–18).



Scheme I

Proposed pathway for the enzymatic synthesis of polysulfated flavonols in *F. chloraefolia*.

Research to elucidate the role of flavonoid sulfation in plant tissues has resulted in the isolation and biochemical characterization of three position-specific flavonol STs from *Flaveria chloraefolia* (19). These enzymes exhibited strict specificity for position 3 of flavonol aglycones and positions 3' or 4' of flavonol 3-sulfate, thus establishing a sequence for the enzymatic sulfation of flavonol polysulfates (Scheme I). Furthermore, the flavonol 3-ST has been purified to homogeneity and partially sequenced, and anti-3-ST antibodies have been produced (20).

In this paper we report the isolation of cDNA clones encoding the flavonol 3- and 4'-STs¶ by anti-3-ST antibody

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Abbreviations: ST, sulfotransferase; SMP-2, senescence marker protein 2.

§To whom reprint requests should be addressed.

¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M84135 and M84136).

Table 1. Substrate specificity of flavonol STs

Substrate	Relative activity, %			
	3-ST	4'-ST	pFST3	pFST4'
Quercetin	58	0	61	0
Isorhamnetin	94	0	90	0
Rhamnetin	100	0	100	0
Kaempferol	48	0	43	0
Quercetin 3-sulfate	0	100	0	100
Isorhamnetin 3-sulfate	0	38	0	33
Tamarixetin 3-sulfate	0	0	0	0
Kaempferol 3-sulfate	0	45	0	39

screening of a cDNA expression library. Comparison of the deduced amino acid sequences of flavonol ST cDNA clones with animal steroid and aryl STs revealed significant sequence similarities, suggesting a common ancestral origin.

MATERIALS AND METHODS

Seeds of *F. chloraefolia* A. Gray (Asteraceae) were kindly provided by A. M. Powell (Sul Ross State University, Alpine, TX). Seeds were germinated in vermiculite on top of potting soil and were further propagated by cuttings.

cDNA Synthesis and DNA Sequencing. Total RNA was isolated from shoot tips of *F. chloraefolia* as described (21). Poly(A)$^+$ RNA was isolated by chromatography on oligo(dT)-cellulose (22), and double-stranded cDNAs were prepared according to the procedure of Gubler and Hoffman (23). After ligation with *Eco*RI linkers, the cDNA was inserted into the *Eco*RI site of the expression vector λZap (Stratagene) (24). A library containing 4×10^6 recombinant phages was obtained, and 3.2×10^5 plaques were screened with specific polyclonal antiserum raised against the flavonol 3-ST (20). Plasmids were rescued from phages that gave a positive signal by using the R408 F1 helper phage (24), and the nucleotide sequences of two full-length cDNA clones (pFST3 and pFST4') were determined on both strands by the dideoxy chain-termination method (25) using oligonucleotide primers. Comparison of the pFST3 and pFST4' cDNA nucleotide sequence with GenBank (release no. 63) and Swiss-Prot (release no. 14) sequence libraries were performed by using the FASTA program package (26).

ST Activity in Extracts of *Escherichia coli*. A culture (5 ml) of the *E. coli* strain XL1-blue containing pFST3 and pFST4' or the vector pBluescript was incubated at 37°C for 3 hr before the addition of the inducer (isopropyl β-D-thiogalactopyranoside) at a final concentration of 1 mM. Incubation was continued for an additional 3 hr. Cells were harvested by centrifugation, washed in 50 mM Tris·HCl, pH 7.5/1 mM EDTA/5 mM dithiothreitol, resuspended, and lysed by sonication in 1 ml of the same buffer. Debris was removed by centrifugation, and the supernatant was desalted by passage through a PD-10 column (Pharmacia). ST activity in bacterial supernatants was determined by monitoring the incorporation of label from 3'-phosphoadenosine 5'-phospho[^{35}S]-sulfate (New England Nuclear; specific activity = 1.5–3.0 Ci/mmol; 1 Ci = 37 GBq) to the flavonol substrate by using a previously described assay (27). An aliquot of the enzyme reaction products was assayed for radioactivity in a liquid scintillation counter, and the remaining fraction was used for the identification of reaction products by cochromatography with reference compounds. TLC was carried out on Avicel cellulose using H_2O or 1-butanol/acetic acid/H_2O (3:1:1, vol/vol) as solvents. Developed chromatograms were visualized in UV light (360 nm) and then autoradiographed on x-ray film.

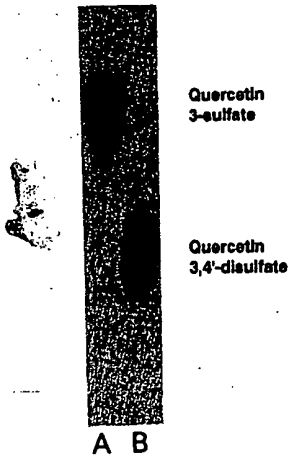

FIG. 1. Photograph of an autoradiogram of the chromatographed reaction products of a crude extract from pFST3-transformed *E. coli* with quercetin as substrate (lane A) and a crude extract from pFST4'-transformed *E. coli* with quercetin 3-sulfate as substrate (lane B).

RESULTS

ST Activity in *E. coli*. The cDNA library was screened (320,000 plaques) with the anti-3-ST antibodies, and 46 positive clones were isolated. Two digestion patterns were obtained when plasmid DNA from positive clones was digested with *Eco*RI. Group I consisted of 6 clones containing a single *Eco*RI fragment of 1200 base pairs, whereas group II consisted of the remaining 40 clones, which contained *Eco*RI fragments of 320 and 950 base pairs. Except for differences in their length, clones belonging to the same group had identical sequences. However, significant differences were observed between group I and group II clones when their sequences were compared.

Group I and II cDNA clones were expressed in *E. coli* in order to demonstrate flavonol ST enzyme activities. A single group I clone containing the longest cDNA insert exhibited ST activity with quercetin but not with partially sulfated flavonoids (Table 1). Since the reaction product cochromatographed with authentic quercetin 3-sulfate and the enzyme showed the same order of substrate preference as the purified 3-ST from *F. chloraefolia* (19) (Table 1), the clone was named pFST3.

All 40 of the group II clones exhibited ST activity with quercetin 3-sulfate as substrate, but not with quercetin, and the reaction product cochromatographed with quercetin 3,4'-disulfate (Fig. 1). To confirm the identity of group II clones as coding for the 3'- or 4'-STs, several substrates were tested. All clones belonging to this group accepted kaempferol 3-sulfate (3' = H, 4' = OH) and isorhamnetin 3-sulfate (3' = OMe, 4' = OH) but did not accept tamarixetin 3-sulfate (3' = OH, 4' = OMe) (Table 1). This corresponds to the same substrate specificity as the purified flavonol 4'-ST isolated from *F. chloraefolia* (19) (Table 1). The clone demonstrating the highest 4'-ST activity (pFST4') was chosen for further characterization. ST activity was not detected with any of the substrates used in this study in *E. coli* cells transformed with the plasmid vector.

Sequence Analysis of pFST3 and pFST4'. DNA sequence analysis of pFST3 and pFST4' revealed the presence of open reading frames coding for proteins of 311 and 320 amino acids, which correspond to molecular masses of 36,442 and 37,212 Da, respectively (Fig. 2). The 5' untranslated region of pFST3 contained 18 nucleotides, whereas that of pFST4' was 66 nucleotides long; 204 and 213 nucleotides separate the stop codon from the poly(A) tail of pFST3 and pFST4', respectively. As is the case for many plant genes (28), no AAUAAA consensus polyadenylylation signal sequence can be found in the vicinity of the poly(A) tail of either clone. Amino acids 25–38 and 160–191 of the deduced sequence of pFST3 were found to match perfectly with previously determined se-

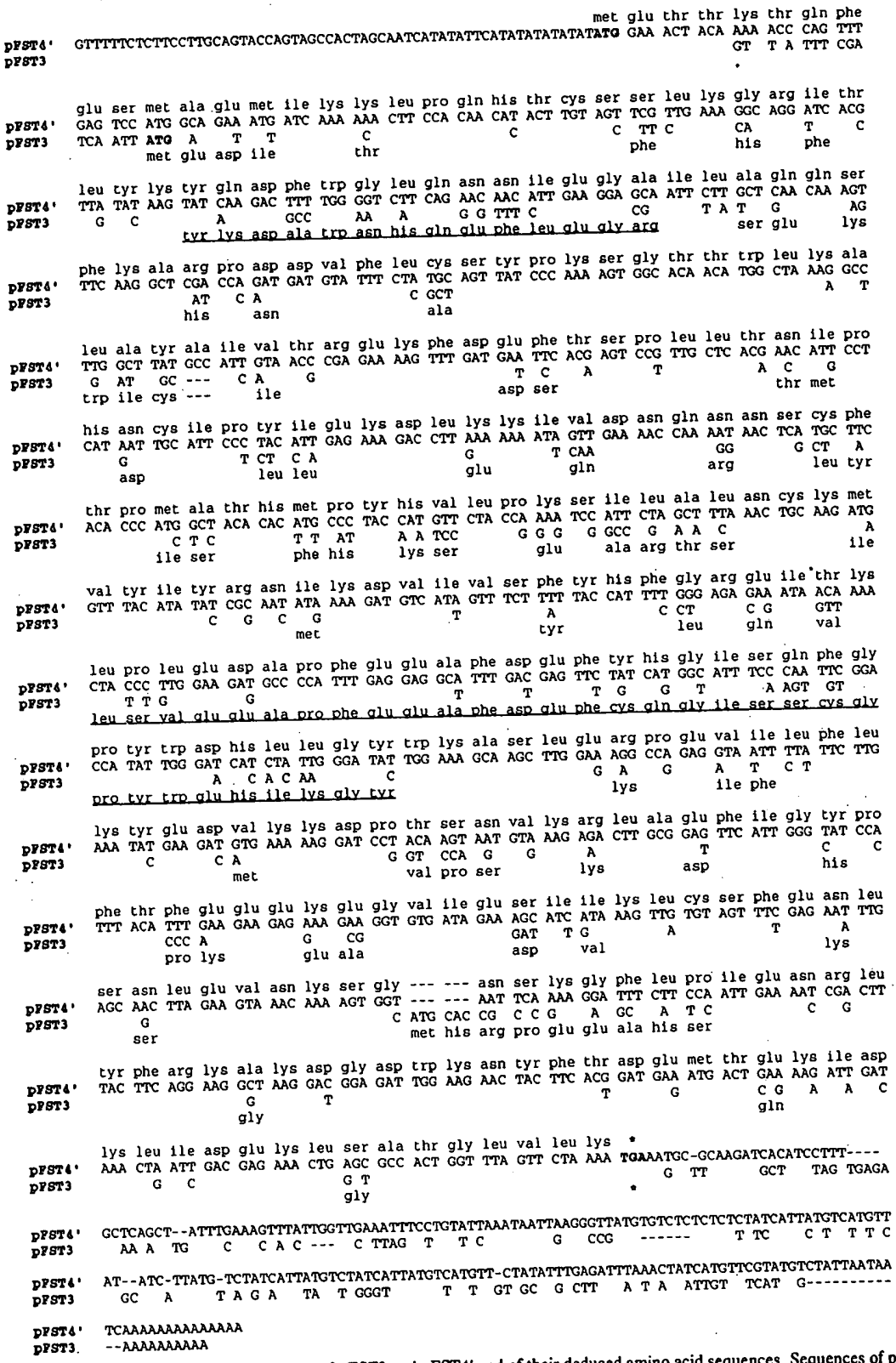

FIG. 2. Alignment of the nucleotide sequences of pFST3 and pFST4′ and of their deduced amino acid sequences. Sequences of pFST3 that are identical to pFST4′ are not shown. The dot indicates the first nucleotide in the sequence of pFST3. Dashed lines indicate deletions in one of the sequences. Underlined sequences of pFST3 match previously sequenced tryptic fragments of *F. chloraefolia*-purified flavonol 3-ST (20).

quences of two tryptic fragments of the flavonol 3-ST (20) (Fig. 2).

Nucleotide and amino acid sequence comparisons of pFST3 and pFST4′ revealed 74% and 69% sequence identity

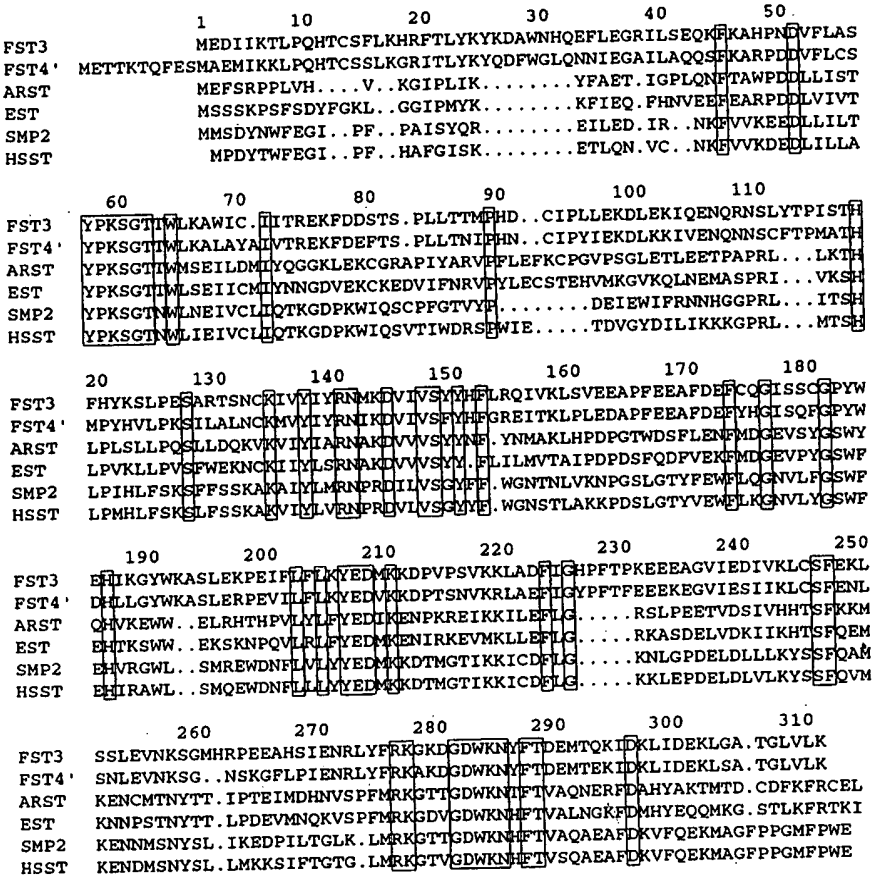

FIG. 3. Amino acid sequence alignment of pFST3 and pFST4′ with rat liver hydroxysteroid ST (HSST) (15), bovine placenta estrogen ST (EST) (17), rat liver aryl ST (ARST) (18), and rat liver senescence marker protein 2 (SMP2) (30). Boxes indicate residues common to the six sequences.

in 1141 nucleotides that overlap and 311 amino acids that overlap, respectively (Fig. 2). The sequence similarity between pFST3 and pFST4′ starts at the beginning of the open reading frame in pFST3. This suggests that the ATG at position 19 in pFST3 is the actual initiation codon in this gene. Examination of the predicted amino acid sequences did not reveal the presence of a signal peptide (29), suggesting a cytoplasmic localization for both STs.

The sequence of pFST3 was used to search DNA and protein sequence data bases. There was 29% identity in a 246-amino acid overlap found with the protein sequence of hydroxysteroid ST from rat liver (15), 31% identity in a 284-amino acid overlap with that of bovine placenta estrogen ST (17), 31% identity in a 192-amino acid overlap with the rat liver senescence marker protein 2 (SMP-2) (30), and 27% identity in a 291-amino acid overlap with the rat liver aryl ST (18). Multialignment of the four ST sequences and of SMP-2 revealed similarities that extended throughout the polypeptides, with four well-conserved regions (Fig. 3).

DISCUSSION

cDNA clones coding for the flavonol 3- and 4′-STs were isolated by antibody screening of an expression library. Both direct and indirect evidence confirms the authenticity of the pFST3 and pFST4′ as coding for the 3- and 4′-STs, respectively. This includes (*i*) the matches between the deduced and directly determined partial amino acid sequence of the 3-ST (Fig. 2); (*ii*) the recognition by anti-3-ST antibodies of a protein expressed in *E. coli* cells harboring pFST3 and pFST4′ plasmids, but not in that harboring the control plasmid; and (*iii*) the position and substrate specificities of the individual ST activities expressed in bacteria harboring the pFST3 and pFST4′ plasmids (Fig. 1 and Table 1). Moreover, the strong sequence similarity between pFST3, pFST4′, and the animal STs is further indication that these two clones encode STs.

The complete nucleotide sequence of pFST3 and pFST4′ revealed an overall positional identity of 69% in 311 amino acid residues between the two proteins. However, the even distribution of the amino acid identities throughout the protein sequences renders difficult the identification of domains that might be determinants for the specificity of the two enzymes. The homology between the two STs suggests that early in the evolutionary process gene duplication followed by sequence divergence has occurred to the point where they no longer function as duplicate genes.

The two STs of *F. chloraefolia* also share sequence similarities with other STs characterized from animal tissues. Identity of 29% and 31% is observed with the hydroxysteroid ST cDNA clone isolated from rat liver and the estrogen ST cDNA clone isolated from bovine placenta. Furthermore, 31% identity is observed between the 3-ST cDNA clone and that of SMP-2 isolated from rat liver. The catalytic function of SMP-2 was unknown when the cloning of its cDNA was reported (30). Recently, Ogura *et al.* (16) reported the isolation of a cDNA clone coding for a hydroxysteroid ST that has 74% homology with the SMP-2 deduced amino acid sequence. This result, together with the homology observed

with the deduced amino acid sequences of the flavonol STs, strongly suggests that SMP-2 is coding for a ST.

The results presented here add a new dimension to comparative studies of STs by providing amino acid sequences from a member of a previously unrepresented phylum. The common evolutionary origin of plant and animal STs becomes evident when we analyze their aligned sequences (Fig. 3). These comparisons may help to define amino acid residues critical to the formation of active STs. The presence of identical amino acid residues is distributed throughout the sequences with four well-conserved regions at positions 45–72, 118–152, 173–210, and 276–296. The stability of sequences in these four regions through evolution suggests that they play an important role in the function of these proteins. In the absence of information on the three-dimensional structure of any of these STs, one can speculate on the role of these regions as being necessary for the appropriate folding of the proteins in order to create the catalytic domain. For instance, the positively charged amino acid residues in these conserved regions could be involved in the binding of the cosubstrate 3′-phosphoadenosine 5′-phosphosulfate. The conservation of the basic structure among these enzymes is further illustrated by comparison of their hydropathy profiles. The hydropathic plots displayed marked similarities in the distribution of hydrophilic and hydrophobic regions (data not shown). This notable similarity suggests that these STs may have similar exposed and buried regions that reflect similar polypeptide folding patterns.

The isolation and characterization of cDNA clones encoding flavonol 3- and 4′-STs summarized here is a step toward elucidation of the key structural features involved in the determination of the strict position and substrate specificities of *F. chloraefolia* STs. Studies of chimeric gene constructs and site-specific alterations of amino acid residues at the ligand-binding regions should provide important information on the structure–function relationships of this important class of proteins. The cloning of the flavonol ST cDNAs will also facilitate studies of the regulation of flavonol sulfation in plants in order to understand the physiological role of these metabolites.

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Exhibit B

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THE ANTIFOULING ACTIVITY OF NATURAL AND SYNTHETIC PHENOLIC ACID SULPHATE ESTERS

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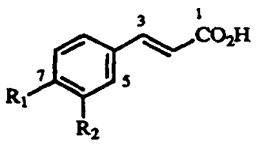
Key Word Index—*Zostera marina*, eelgrass, marine biofouling, *p*-(sulphooxy) cinnamic acid, sulphate esters, phenolic acids.

Abstract—*p*-(sulphooxy) Cinnamic acid was isolated as a natural product for the first time from the seagrass *Zostera marina* (eelgrass) and was found to prevent attachment of marine bacteria and barnacles to artificial surfaces at non-toxic concentrations. Analogous synthetic sulphate esters had similar antifouling properties, while the non-sulphated phenolic acid precursors were ineffective. The antifouling properties of phenolic acid sulphates are consistent with an emerging pattern of biological activity exhibited by other sulphate esters isolated from a variety of marine organisms, and their low toxicity offers promise for the development of environmentally benign antifouling agents to protect structures in aquatic environments.

INTRODUCTION

Phenolic compounds are widely distributed in marine plants and a number of biological functions have been attributed to them [1–5]. Examples of these natural products isolated from *Zostera marina* L. (eelgrass) to date include flavone sulphates [3] (the 7-sulphates of luteolin, diosmetin, apigenin, chrysoeriol and the 7,3′-disulphate of luteolin) and non-sulphated phenolic acids [1. 6] (*p*-coumaric, ferulic, caffeic, vanillic, gallic, protocatechuic, gentisic, 4-hydroxybenzoic and *o*-pyrocatechuic acids). Previous studies with leaf extracts suggest that phenolic constituents of eelgrass may inhibit amphipod grazing, microbial growth [7] and photosynthetic carbon uptake in epiphytic diatoms [8]. It has been suggested that phenolic acids may also confer resistance to the so-called 'wasting disease' blamed for the catastrophic die-off of eelgrass in the North Atlantic during the 1930s [9]. Although seasonal variation in total phenolic content of eelgrass leaves does not support a strong antifouling role for these compounds in nature [10], crude methanolic extracts of eelgrass have been found to inhibit the attachment of marine bacteria, diatoms, barnacles and polychaetes to artificial surfaces (Zimmerman, unpublished).

The goal of this study was to search for and characterize compounds present in extracts of *Z. marina* leaves that act to prevent the attachment of marine bacteria, epiphytic algae and invertebrates to submerged surfaces without being indiscriminate toxins. Here we report the first isolation of naturally occurring *p*-(sulphooxy) cinnamic acid (*p*-coumaric acid sulphate, **1**), and compare its antifouling properties with those of non-sulphated phenolic acids and synthetic phenolic acid *p*-sulphate esters.



	R_1	R_2
1	OSO_3H	H
2	OH	H
3	OH	OMe
4	OH	OH
5	OSO_3H	OSO_3H
6	OSO_3H	OH
7	OH	OSO_3H
8	OSO_3H	OMe

RESULTS AND DISCUSSION

The major antifouling component isolated from the methanolic extract of eelgrass shoots was shown by a ^{13}C APT NMR spectrum to contain six methine and three quaternary carbons, while the ^1H NMR spectrum indicated two aromatic proton doublets and two vinylic proton doublets. These data indicated a *para*-substituted *trans*-cinnamic acid (Experimental). The unknown substituent was suspected to be a *p*-sulphooxy group when it was noted that the compound decomposed to *p*-coumaric

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acid (2) during HPLC purification using 0.1% aqueous TFA. A negative high resolution fast atom bombardment mass spectrum (HRFABMS) of purified natural 1 gave the predicted molecular ion. Treatment of natural 1 with diazomethane yielded the expected methyl *p*-(sulphooxy) cinnamate (methyl C and H at δ50.8 and 3.72) consistent with NMR spectra and MS $[M]^+$ ion. Final confirmation of structure was obtained by matching the NMR data of natural 1 purified from eelgrass with those of 1 synthesized from 2 and $ClSO_3H$.

The sulphate esters of the three cinnamic acid derivatives known to occur in eelgrass [2, ferulic (3) and caffeic (4) acids] were synthesized in order to determine whether the sulphate moiety was primarily responsible for the antifouling property of 1. Since the *p*-sulphate ester was acid-labile, synthesis was performed under basic or neutral conditions to the extent possible. Acid hydrolysis of the sulphate bond may explain previous failures to detect these compounds in eelgrass extracts by chromatographic separations performed at low pH [4]. The reaction of 4 with $ClSO_3H$ gave a mixture of the disulphate (5) and the two monosulphates (6, 7). Although the monosulphates were not resolved by the TLC or HPLC conditions employed, the presence of the two isomers was apparent from the ^{13}C NMR spectrum showing 18 carbons and the expected molecular ion observed by negative HRFABMS.

Antifouling dose-effectiveness of the three synthetic phenolic acid sulphates (1, 5 and 8) against a marine bacterium was statistically identical to natural 1 isolated from eelgrass (Fig. 1); the aggregate EC_{50} value was approximately 10 $\mu g\,cm^{-2}$. The quantitative dose response determined by linear regression was highly significant (attached bacterial density $= 76 - 0.21 \cdot \log$ dose, $r^2 = 0.83$, $P < 0.01$). In contrast, the non-sulphated phenolic acids (2–4) showed no significant antifouling activity at concentrations 60-fold higher than the EC_{50} dose for the sulphate esters (data not shown). Therefore, the presence of the sulphate ester was necessary for the antifouling function. LD_{50} concentrations remain undefined because these compounds could not inhibit growth of the bacteria in liquid culture at concentrations at least three orders of magnitude higher than those required to inhibit attachment. Only the sulphate ester of ferulic acid (8) has been examined for its ability to inhibit attachment of barnacle (*Balanus amphitrite*) cyprids and its effectiveness was similar to that observed for marine bacteria, again without toxicity (Fig. 2).

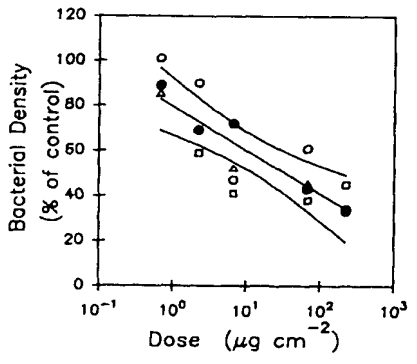

Fig. 1. Antifouling dose–response of natural *p*-(sulphooxy) cinnamic acid (1) ( ), synthetic *p*-(sulphooxy) cinnamic acid (1) (○), *p*-(sulphooxy) ferulic acid (8) (△) and 3,4 (disulphooxy) caffeic acid (5) (□), relative to control slides, against *Acinetobacter* sp. Lines indicate linear regression of log-transformed data and 99% confidence limits.

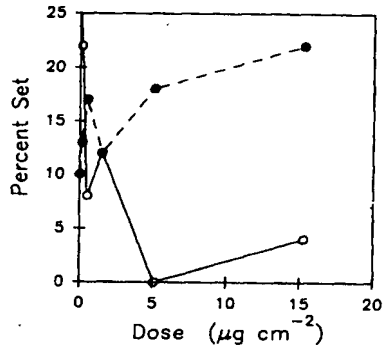

Fig. 2. Antifouling dose–response curve for synthetic *p*-(sulphooxy) ferulic acid (8) (○) against barnacle (*B. amphitrite*) larvae in laboratory assays and solvent controls (●).

The antifouling properties of phenolic acid sulphates are consistent with an emerging pattern of biological activity attributed to sulphate esters from a variety of marine organisms. A number of polycyclic phenolic sulphates isolated from several species of crinoids have effectively deterred predation by fish [11]. Similar studies of steroid sulphates isolated from sponges [12–14] demonstrated that the sulphate group was required for antimicrobial activity of these compounds. Although the antifouling mode of action of phenolic acid sulphates is currently unknown, these compounds are highly water-soluble and a solution-active mechanism is possible. The extremely low toxicity of the phenolic acid sulphates also suggests that these compounds may provide an environmentally acceptable means to control biofouling in aquatic environments.

EXPERIMENTAL

Plant material. Emergent shoots of the seagrass *Z. marina* L. were collected by scuba divers from a subtidal bed(5–7 m deep) near Del Monte Beach, in Monterey Bay, California (36° 36′ 15″ N, 121° 53′ 10″ W) in March (dry wt 575 g) and October (dry wt 1700 g) 1990.

Extraction and isolation. The combined dry residue from 3 MeOH extractions (20°) of freshly dried ground eelgrass leaves was extracted with H_2O prior to being partitioned between hexanes and 10% aq. MeOH; the MeOH phase was then diluted to 40% aq. MeOH and extracted with CH_2Cl_2. Bioassays of these frs indicated antifouling activity was principally localized in the H_2O extract. Lyophilization of the aq. extract gave a hygroscopic solid that was sepd batchwise into 3 coloured

bands on a Sephadex LH-20 column (42 × 3.2 cm OD, MeOH). The compound with antifouling activity, which was concd in the middle yellow band, was identified as **1** after removal of inorganic salts and other impurities by HPLC. Quantitative estimates of yield were not calcd from the March collection because preliminary experiments were performed with various frs that resulted in acid decomposition of **1**. The October collection, however, yielded 66 mg of **1** from 1700 g dry biomass.

Chromatography. Final purification of all phenolic acid sulphates (natural and synthetic) was carried out by HPLC [Regis ODS irregular column, 25 cm × 10 mm ID; RI detector; 90% aq. MeOH solvent (100% H_2O for **5–7**); 1000 psi]. TLC R_f values [silica gel; BuOH–HOAc–H_2O (4:1:1); UV detection]: **1** (0.63); **2** (0.83); **3** (0.75); **4** (0.80); **5** (0.29); **6** and **7** (0.61); **8** (0.58).

Antifouling bioassays. Frosted ends (4.5 cm^2) of glass microscope slides were treated with candidate frs or purified compounds dissolved in MeOH and challenged against a clone of *Acinetobacter* sp., a fouling marine bacterium isolated from the surface of eelgrass leaves. Control slides were treated with MeOH solvent only. Other than air evapn of the MeOH solvent, no attempt was made to stabilize or otherwise prevent the dissolution of material from the treated surfaces once they were immersed in seawater. Slides were placed into 50 ml screw-cap plastic tubes containing 30 ml of sterile, 0.2 μm filtered seawater and an inoculum of bacteria from a log-phase liquid culture (final bacterial concn was *ca* 10^6 cells ml^{-1}). Tubes were capped and placed horizontally on a rotary shaker with the treated surface facing down to prevent bacteria from simply settling out on the slides. Thus, bacteria attaching to the surface did so against gravity and the randomizing force of H_2O motion. Slides were removed at 20-min intervals, stained with Hoechst (No. 2287, Sigma Co.) and cell densities in the frosted regions were enumerated with the aid of epifluorescence microscopy (1000 ×). Attached bacterial densities on treated slides were normalized to control slides at each time point. Mean density (relative to MeOH control) was calcd for each concn from all data in the 4 hr time series.

Barnacle attachment assays were performed in plastic Petri dishes treated with **8** and control solvent (MeOH). Dishes were then filled with FSW and competent cyprids of *B. amphitrite* were added. As with the bacterial assays, no attempt was made to stabilize or otherwise prevent the dissolution of material from the treated surfaces. After 24 hr, the number of attached cyprids in each dish was determined and normalized to the number added initially. Ten replicate dishes were analyzed for each concn.

Synthesis of **1**. $ClSO_3H$ (0.2 ml) was added dropwise to **2** (200 mg) in pyridine (0.5 ml) with stirring at 20°. Ice H_2O was added and the acidic aq. mixt. extracted with Et_2O, basified, extracted with Et_2O, and H_2O removed under vacuum. The residue was triturated with H_2O, neutralized, dried under vacuum and triturated with MeOH. The MeOH-soluble residue purified by HPLC gave 187 mg **1** (63%). Similar yields were obtained for **5–8**.

Spectral data. All mass spectra were negative HRFAB. Matrix: thioglycerol/glycerol (only glycerol for **5–7**). NMR * assignments interchangeable.

Natural **1**: $[M-1]^-$ *m/z* 242.9963, $C_9H_7O_6S$, Δ 0.0 mmu. APT and ^{13}C NMR (62.5 MHz, CD_3OD-D_2O): δ 176.5 (*s*, C-1), 153.5 (*s*, C-7), 140.8 (*d*, C-3), 134.3 (*s*, C-4), 130.2 (2C, *d*, *C-5), 125.8 (*d*, C-2), 122.9 (2C, *d*, *C-6); ^1H NMR (300 MHz, CD_3OD-D_2O): δ 7.59 (2H, d, J = 8.7 Hz, *H-5), 7.34 (1H, *d*, J = 16.2 Hz, H-3), 7.27 (2H, *d*, J = 8.4 Hz, *H-6), 6.44 (1H, *d*, J = 16.2 Hz, H-2). Note: the chemical shift for H-3 depends strongly on the ionization state of the carboxyl group.

Methyl ester of natural **1**: $[M-1]^-$ *m/z* 257.0118, $C_{10}H_9O_6S$, Δ0.2 mmu of calcd. ^{13}C NMR (75 MHz, CD_3OD): δ167.8, 154.5, 144.3, 130.7, 128.9 (2C), 121.2 (2C), 116.5, 50.8; ^1H NMR (300 MHz, CD_3OD): δ7.65 (*d*, J = 15.9 Hz), 7.57 (*d*, J = 8.7 Hz), 7.31 (*d* J = 8.7 Hz), 6.45 (*d*, J = 15.9 Hz), 3.72 (*s*).

Synthetic **1**: $[M-1]^-$ *m/z* 242.9958, $C_9H_7O_6S$, Δ 0.5 mmu of calcd. NMR spectra same as natural **1**.

Compound **5**: $[M-2H+Na]^-$ *m/z* 360.9285, $C_9H_6NaO_{10}S_2$, Δ 1.5 mmu of calcd. ^{13}C NMR (62.5 MHz, D_2O): δ176.5, 144.9, 144.3, 140.3, 135.3, 127.4, 124.4, 123.3, 123.1; ^1H NMR (250 MHz, D_2O): δ7.81 (*br s*), 7.62 (*br s*), 7.45 (*d*, J = 16.0 Hz), 6.61 (*d*, J = 16.0 Hz).

Mixture of **6** and **7**: $[M-1]^-$ *m/z* 258.9888, $C_9H_7O_7S$, Δ 2.4 mmu of calcd. ^{13}CNMR (62.5 MHz, D_2O): δ177.0, 176.7, 151.0, 149.4, 141.2, 140.9, 140.1, 135.4, 128.9, 128.1, 127.2, 125.7, 124.1, 123.5, 123.0, 121.4, 118.8, 117.0; ^1HNMR (250 MHz, D_2O): δ7.74 (*s*), 7.59 (*d*, J = 2.0 Hz), 7.56 (*s*), 7.42–7.35 (*m*), 7.30 (*d*, J = 3.2 Hz), 7.22 (*d*, J = 1.9 Hz), 7.18 (*d*, J = 2.0 Hz), 7.15 (*d*, J = 2.0 Hz), 7.03 (*d*, J = 8.4 Hz), 6.53 (*d*, J = 18.3 Hz), 6.41 (*d*, J = 14.8 Hz).

Compound **8**: $[M-1]^-$ *m/z* 273.0083, $C_{10}H_9O_7S$, Δ 1.4 mmu of calcd. ^{13}CNMR (62.5 MHz, CD_3OD): δ175.6, 153.1, 143.5, 140.2, 134.8, 126.3, 123.5, 121.2, 112.5, 56.6; ^1HNMR (250 MHz, CD_3OD): δ7.40 (*d*, J = 8.3 Hz), 7.30 (*d*, J = 15.9 Hz), 7.14 (*d*, J = 1.9 Hz), 7.02 (*dd*, J = 8.4, 1.9 Hz), 6.40 (*d*, J = 15.9 Hz), 3.83 (*s*).

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